

**APPLICATION**

**FOR**

**UNITED STATES LETTERS PATENT**

**BY**

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**FOR**

**METHOD AND DEVICE FOR DETECTION OF APO A, APO B**

**AND THE RATIO THEREOF IN SALIVA**

# METHOD AND DEVICE FOR DETECTION OF APO A, APO B AND THE RATIO THEREOF IN SALIVA

## Background of the Invention

5 The present invention is generally in the field of assays for apolipoproteins in saliva.

This application claims priority to U.S.S.N. 60/124,562 filed March 16, 1999.

10 Coronary artery disease (CAD) is the leading cause of morbidity and mortality in most developed countries. Numerous markers and tests for identifying individuals at risk are available, among them blood tests for lipid markers such as total cholesterol and cholesterol bound to various circulating proteins. Based on the outcome of such testing, appropriate prophylactic or therapeutic measures including dietary modification and exercise can be 15 initiated to forestall or reverse progression to more severe CAD.

Plasma lipoproteins are carriers of lipids from the sites of synthesis and absorption to the sites of storage and/or utilization. Lipoproteins are spherical particles with triglycerides and cholesterol esters in their core and a layer of phospholipids, nonesterified cholesterol and apolipoproteins on the surface. 20 They are categorized into five major classes based on their hydrated density as very large, triglyceride-rich particles known as chylomicrons (less than 0.95 g/ml), very low density lipoproteins (VLDL, 0.95 to 1.006 g/ml), intermediate-density lipoproteins (IDL, 1.006 to 1.019 g/ml), low-density lipoproteins (LDL, 1.019 to 1.063 g/ml) and, high-density lipoproteins (HDL, 1.063 to 1.210 g/ml). 25 (Osborne and Brewer, Adv. Prot. Chem. 31:253-337 (1977); Smith, L.C. et al. Ann. Rev. Biochem., 47:751-777 (1978)).

Apolipoproteins are protein components of lipoproteins with three major functions: (1) maintaining the stability of lipoprotein particles, (2) acting as cofactors for enzymes that act on lipoproteins, and (3) removing lipoproteins 30 from circulation by receptor-mediated mechanisms. The four groups of apolipoproteins are apolipoproteins A (Apo A), B (Apo B), C (Apo C) and E

(Apo E). Each of the three groups A, B and C consists of two or more distinct proteins. These are for Apo A: Apo A-I, Apo A-II, and Apo A-IV, for Apo B: Apo B-100 and Apo B-48; and for Apo C: Apo C-I, Apo C-II and Apo C-III. Apo E includes several isoforms. Each class of lipoproteins includes a variety 5 of apolipoproteins in differing proportions with the exception of LDL, which contains Apo B-100 as the sole apolipoprotein. Apo A-I and Apo A-II constitute approximately 90 percent of the protein moiety of HDL whereas Apo C and Apo E are present in various proportions in chylomicrons, VLDL, IDL and HDL. Apo B-100 is present in LDL, VLDL and IDL. Apo B-48 resides 10 only in chylomicrons and so called chylomicron remnants (Kane, J.P., Method. Enzymol. 129:123-129 (1986)).

Total plasma or serum cholesterol (C) has traditionally been the primary screening and indicator of CAD, but the emphasis has recently shifted to serum lipoprotein profiles including HDL, LDL, VLDL, lipoprotein A and particularly 15 to the LDL/HDL or Total C/HDL ratios which have shown better correlations with incidence and severity of CAD. In contrast to the atherogenic potential of LDL, VLDL and VLDL remnants, HDL are inversely correlated with CHD, so that individuals with low concentrations of HDL-C have an increased incidence of CHD (Gordon, T. et al., Am. J. Med., 62:707-714 (1977); Miller, N.E. et al., 20 Lancet, 1:965-968 (1977); Miller, G. J. and Miller, N.E., Lancet, 1:16-19 (1975)).

A large number of manual and automated methods are available for screening and monitoring of these markers. All of these tests, however, require either venous blood drawn by syringe or, in some cases, capillary blood obtained 25 by needle prick. Both methods are invasive and unpleasant to many individuals and are best performed by trained professional personnel, preferably in doctor's office, to minimize erroneous results. Handling and disposal of blood products also involves potential hazards from infectious agents and pathogens.

It is thus highly desirable to provide safer alternative specimens not 30 requiring invasive procedures. Furthermore, the ideal analytical method or

device should provide rapid and reliable results for point of collection ("POC") diagnosis at low cost.

Most analytes that appear in serum also appear in saliva, but at levels that are a fraction of their level in serum. The transport of an analyte into saliva 5 can be by intracellular (diffusion or passive transport) or extracellular (active transport) transport. Materials that are lipid soluble enter saliva by diffusion through cellular compartments. Haekcel, Ann. N.Y.Acad. Sci. 694, 128-142 (1993).

10 Saliva has not been exploited as a diagnostic fluid because of the many problems associated with adapting it to assay form. For example, it is difficult to collect sufficient sample: Most tests require collection of at least 1 ml of saliva because there is considerable loss during filtration and handling. This requires an average of 3-5 minutes of salivation, which most people are not willing to do. The average flow rate for 95% of young men is 0.35-0.38 ml/min.

15 (K. Diem, et al (ed) Scientific Tables (Ciba-Geigy Pharmaceuticals 1970) p. 643. Moreover, the handling of saliva samples to prepare them for assay is both tedious and unpleasant. Saliva generally has to be filtered to remove the mucopolysaccharides and allow flow and handling. Available collection devices utilize cotton pads to absorb saliva in the mouth. The pad thus acts to collect 20 and process the saliva, preparing it for assay. The pad is then placed in a volume of fluid containing preservatives and shipped to the laboratory for analysis. The preservative fluid prevents quantitation by making it impossible to know how much saliva, if any, was collected and added to the preservative.

25 When the device reaches the lab the technicians must remove the pad and mucopolysaccharides either by centrifugation or filtration. This is a time consuming and unpleasant job. The small amount of saliva sample and low level of analyte in saliva usually means that the saliva sample cannot be analyzed by an autoanalyzer, but must be assayed in a high sensitivity Elisa or RIA, both of which are labor-intensive tests.

30 Many studies of saliva have shown that the levels of analytes vary with the secreting gland and the method of collection (e.g. stimulated flow versus

normal flow). For reviews see Saliva as a Diagnostic Fluid (D. Malmud and L. Tobak, Eds., Ann. N.Y. Acad. Sci. Vol. 694 D (1993) and J.O. Tenvuuo (ed) Human Saliva: Clinical Chemistry and Microbiology (CRC Press Inc. 1989) vol. I and II). Thus, one presumes that the significant variations in lipid levels 5 reported in saliva are in large part due to collection method. Levels of cholesterol are also low, with cholesterol levels of about 1/400 and about 1/50 of that seen in serum. Bronislaw, et al., "Lipids of Saliva and Salivary Concretions," in Human Saliva: Clinical Chemistry and Microbiology (CRC Press Inc. 1989) vol. II, 121-145). Thus the level in an individual sample is too 10 low for conventional serum assays in the routine assay of lipids in saliva, therefore either requiring the use of sensitive immunoassays or a larger quantity of saliva. J.C Touchstone, et al., "Quantitation of Cholesterol in Biological. . ." in Adv. Thin Layer Chromatogr., Proc. Bienn. Symp. Meeting Date 1980, (Wiley & Sons 2<sup>nd</sup> ed. 1982) measured total cholesterol and lipids. Moreover, 15 there is a variation in levels depending on the time of day and from day to day (less than 8%), with levels highest in morning specimens and lower throughout the day, suggesting that saliva testing of cholesterol be done at the same time of the day.

Another problem with using saliva is that saliva is heavily contaminated 20 with the oral flora. Available collection devices provide high levels of preservatives to retard growth of bacteria but, unless the sample is carefully preserved (e.g. by freezing), samples often become putrefied and laboratory technicians avoid processing saliva. Furthermore, high levels of preservatives can interfere in many assays. Saliva also contains many proteins and enzymes 25 of both salivary and bacterial origins. Over time these enzymes and proteins can interact with the analytes of interest and make the assay of some analytes impossible. Thus, as a rule, stored samples cannot be expected to yield accurate results unless the storage additives and conditions are optimized for the analyte.

The literature reports that, while cholesterol is present in saliva, the 30 levels vary greatly. For example, 5.6 mg/L average was reported by B. Larsson, et al, "Lipids in Human Saliva" in Archs. Oral. Biol. 41(1), 105-110 (1996); 15

mg/L average was reported for both the parotid and submandibular glands saliva output by Slomiany, et al, J. Dent. Res. 61(1), 24-27 (1983); and 69 mg/L was reported by Rabinowitz, et al, Arch. Oral. Biol. 20(7), 403-406 (1975).

As noted above, the ratio of LDL:HDL ratio is an established predictor 5 of the risk of coronary artery disease. The recent NCEP guidelines call for use of ratio rather than total cholesterol. It has been reported that men with acceptable total cholesterol levels but ratios of LDL:HDL above 3.5 were 50% more likely to have coronary heart disease than their counterparts with lower ratios. It is a matter of time before total cholesterol is supplanted by ratios.

10 Immunoassays for lipoproteins associated with HDL and LDL have been shown to correlate with the measurement of cholesterol ratios in these two fractions. N. Rifai, et al (ed) Laboratory Measurement of Lipids, Lipoproteins and Apolipoproteins (AACC Press 1994) p. 114. The results correlate with the methods where HDL and LDL fractions are physically separated and measured 15 (Laboratory Measurement of Lipids, Lipoproteins and Apolipoproteins. 1994. N. Rifai and R. Warnick Eds. AACC Press.)

It is not known if the proteins with which salivary cholesterol is associated are the same as those in serum, i.e. ApoAI and ApoBII. It is clear from all studies (Belmont) (Mandel, et al, Arch. Oral. Biol. 14(2), 231-233 20 (1969)) that salivary lipids are secreted by the glands in conjunction with lipoproteins(s). Slomiany et al also demonstrated that the lipids in saliva are associated with proteins. There is no published literature, however, on the origin 25 of the lipids or their physical state in saliva" (Larsson et al). Thus, from the early literature, it is not clear whether the salivary lipids are synthesized de novo in the salivary glands or are derived from serum; and, if they are serum derived, if the salivary apolipoproteins are the same as the apolipoproteins associated with LDL and HDL in serum. There are other salivary glycoproteins also associated with the lipid. It is not clear from the literature whether the structure 30 of the lipid particles in saliva is the same as those in serum and whether the conformation of the apolipoproteins is the same in saliva as in serum. Rabinowitz suggests that lipids secreted by the glands are secreted associated

with lipoprotein. He demonstrated that the lipid levels drop in stimulated saliva but retain the same ratio to one another. Larsson reports that salivary lipoprotein fractions are of much higher density than serum lipoproteins and concludes that the salivary lipids are differently aggregated.

5      Various studies have indicated that the saliva levels of cholesterol show a gross correlation with serum cholesterol levels (Lochner, A. Dissertation Abstract International (1985) Vol. 46, #5B). It has also been observed that there is a positive correlation between persons with hypercholesterolemia. Slomiany, et al, Arch. Oral. Biol. 27(10), 803-808 (1982) and Murty, et al, RCS Med. Sci.

10     10(5), 359 (1982). The paucity of studies correlating serum and saliva cholesterol may be due to the fact that the available methods for assaying cholesterol and thus correlating serum and saliva have been too insensitive. The enzymic and chromatographic methods of detecting cholesterol rely on high levels not available in saliva. Thus, these methods require large amounts of

15     saliva and studies on lipids have generally been done on pools. Measurement of cholesterol in saliva is further complicated because saliva contains high amounts of peroxidase, an enzyme component of some cholesterol assays.

It is therefore an object of the present invention to provide a non-invasive, non-instrumental, accurate, simple and cost-effective means for 20 determination of a marker for CAD, HDL, LDL, and/or the ratio of LDL:HDL.

### Summary of the Invention

A method has been developed to detect the levels of apolipoproteins A-1 and B in saliva, which is correlated with the levels of HDL and LDL in serum, 25 respectively. In unstimulated saliva, the ratio of Apo A to Apo B is correlated with the ratio of HDL to LDL in serum. In stimulated saliva the levels of Apo B normalized to albumin correlate with both serum Apo B and serum LDL. The high degree of correlation in combination with a simple, quick test that can be performed at the site of collection provides a cost effective, patient friendly 30 means to monitor an individual's risk of heart disease. In the preferred embodiment, saliva production is stimulated by means such as breath mint or

tart solution (such as lemon) and the effect of dilution controlled by reference to albumin. In the most preferred embodiment, the assay is an immunoassay performed using the Serex laminated strip format as described in U.S. patent Nos. 5,710,009, 5,500,375, and 5,451,504. These strips are advantageous since 5 they serve as the collection and assay device, greatly simplifying handling, as the sample is applied directly to the strip test and processed as an integral part of the analytical procedure. This method requires less than 200 microliters, which should be available in the average person's mouth at any time. Additional saliva production can be obtained, however, using breath mints or a tart juice such as 10 lemon juice. The assay of saliva at POC will eliminates the need for preservatives to store the sample and entirely avoids the problem with contamination by oral flora, since the assay can be completed within 10 minutes of saliva collection.

#### **Brief Description of the Drawings**

15 Figure 1 is a graph of the absorbance of saliva samples assayed by ELISA for the presence of HDL, using serum and Apo A1 at different dilutions (1x 1:10, 1:100) as controls.

*a* <sup>A</sup> Figure <sup>2a</sup> is a graph of the absorbance of saliva samples assayed by ELISA for the presence of LDL, using serum and Apo B as different dilutions 20 (1x, 1:10, and 1:100) as controls.

*a* <sup>2B</sup> Figure <sup>2a</sup> is a graph of Apo B (mg/dl) relative to Apo A1 (mg/dl) in <sup>2C</sup> lemon stimulated saliva. Figure <sup>2b</sup> is a graph of Apo B (mg/dl) relative to Apo a1 per mg of albumin in lemon stimulated saliva.

25 Figure 3a is a graph of color density in a strip immunoassay for LDL for LDL in serum at dilutions of 1:100, 1:75, 1:25 and 1:10. Figure 3b is a graph of color density in a strip immunoassay for HDL for HDL in serum at dilutions of 1:1,200, 1:600, 1:100, and 1:10.

30 Figure 4a is a graph of the correlation of the ratio of Apo B to Apo A in serum. Figure 4b is a graph of the correlation of the ratio of Apo B in serum measure by the ELISA described herein compared to the Roche commercially

available Cobas Mira assay. Figure 4c is a graph of the correlation of the ratio of Apo B to Apo A in lemon stimulated saliva.

### **Detailed Description of the Invention**

5 It has now been demonstrated that ApoA and ApoB are both present in saliva, but that these proteins are not detectable by electrophoresis or immunoassay except in very fresh samples, presumably due to degradation of the lipoproteins by saliva enzymes or bacteria or both. This explains why other studies have not observed these proteins; since they detected cholesterol by 10 enzymic methods, and in the time required for this the proteins were degraded.

10 **I. Reagents for Detection of Apolipoproteins**

Antibodies are known in the literature and available from commercial sources and from the ATCC.

#### Antibodies to Apo B

##### *Antibodies to Pan B*

15 D<sub>6</sub> MAb is an antibody with equal binding and high affinity for all Apo B-containing lipoproteins in human plasma, as described by Koren, E. et al., *Biochim. Biophys. Acta*, 876:91-100 (1986); Koren, E. et al., *Biochim. Biophys. Acta*, 876:101-107 (1986), specifically including Apo B-48 and Apo B-100. D<sub>6</sub> binds to an epitope localized at the amino terminal half of Apo B and recognizes 20 both B-48 and B-100.

##### *Antibodies to Apo B-100*

Conventional ways of producing Monoclonal antibodies to Apo B-100 include immunization of mice with LDL. This approach is convenient because 25 it is relatively simple to isolate LDL. However, Monoclonal antibodies produced using LDL as an immunogen tend to be sensitive to conformational changes of Apo B-100 caused by variations in the lipid composition of LDL particles. For example, Apo B-100 epitopes are less reactive with a number of anti-Apo B Monoclonal antibodies due to the presence of various amounts of 30 triglycerides (Keidar, S. et al., *Metabolism*, 39: 281-288 (1990); Galeano, N.F. et al., *J. Biol. Chem.*, 269:511-519 (1994); Harduin, P. et al., *Arterioscl.*

Thromb., 13: 529-535 (1993)).

PCT PCT/US95/08331 "Antibodies to Lipoproteins and Apolipoproteins and Methods of Use Thereof" by Oklahoma Medical Research Foundation describes antibodies that provide selective recognition of LDL and high and invariable reactivity with LDL particles, irrespective of possible variations in their lipid composition and/or conformation, that is, an antibody which recognizes a stable, conformation-independent epitope which is uninfluenced by the lipid content and which is equally expressed in all LDL particles, but inaccessible in VLDL and chylomicrons. HB<sub>3</sub>cB<sub>3</sub> binds to the epitope near the T2 carboxy terminal region of B-100, exclusively, and does not recognize B-48. The epitope recognized by HB<sub>3</sub>cB<sub>3</sub> may be conformationally changed or masked by lipids and/or other apolipoproteins present in VLDL. Chylomicrons are not recognized by HB<sub>3</sub>cB<sub>3</sub> because they lack Apo B-100. The HB<sub>3</sub>cB<sub>3</sub> antibody and LDL-binding fragments derived therefrom, can be used as an LDL-specific binding molecule in all of the compositions and methods described herein because of its specificity for LDL and lack of cross-reactivity with other lipoproteins.

Two other LDL specific Monoclonal antibodies are described by Milne, R. et al., J. Biol. Chem., 264:19754-19760 (1989); and WO 93/18067 by La Belle, et al. and La Belle, M. et al., Clin. Chim. Acta, 191:153-160 (1990) (8A2.1 and 4B5.6).

#### Antibodies to Apo A-I

Two antibodies to Apo A-I are also described in PCT PCT/US95/08331 by OMRF which both bind to HDL with a high affinity and show negligible reactivity with any other lipoprotein density class. The two anti-Apo A-I Monoclonal antibodies, AIbD<sub>5</sub> and AIbE<sub>2</sub>, bind to sterically distant epitopes since they do not compete with each other in their binding to either delipidized and purified Apo A-I or intact HDL particles. Both Monoclonal antibodies to Apo A-I bind with high affinity to delipidized Apo A-I and to HDL and show negligible or no binding to LDL, VLDL, chylomicrons and Apo A-II, C-III and E

#### Antibodies to Apo A-II

A monoclonal antibody to Apo A-II which binds with high affinity to HDL and is capable of removing all the HDL particles containing Apo A-II (LP-A-I:A-II particles) from plasma or serum, leaving the HDL particles without 5 Apo A-II (LP-A-I particles) intact, CdB<sub>5</sub>, is described by Koren, E. et al., Arteriosclerosis, 6:521a (1986); Alaupovic, P. et al., J. Lipid Res., 32:9-19 (1991).

#### Antibodies to Apo C-III

An MAb to Apo C-III, XbA<sub>3</sub>, which is useful in quantification of VLDL 10 particles is described by Koren, E. et al., Atherosclerosis, 95:157-170 (1992).

#### Antibodies to Apo E

Two Monoclonal antibodies to Apo E are described by Koren, E. et al., Atherosclerosis, 95:157-170 (1992). One of them, EfB<sub>1</sub>, binds preferably to Apo E associated with VLDL which are precipitated by heparin whereas the 15 other (EfD<sub>3</sub>) binds predominantly to Apo E in HDL which are not precipitated by heparin treatment of a sample.

### **II. Sample Preparation**

In a preferred embodiment, saliva is collected, filtered through cotton or a similar pore size filter to remove mucopolysaccharides, and kept chilled to 4°C 20 until assayed. Preferably the sample is assayed within three hours of collection. Preservatives and protease or other enzyme inhibitors can be added to the sample.

### **III. Simultaneous Sample Collection, Preparation and Assay**

In an alternative embodiment, an integrated collection and assay device 25 can be used. As described in PCT PCT/US98/16256 "*Integrated Collection and Assay Device for Saliva and Blood*" by Serex, Inc., this device can include a fluid collector, a processing and metering pad and/or filter, and one or more assay strips. The fluid collector can be adapted for collection of saliva or blood. The assay strips can be any type presently used. Nitrocellulose is a preferred 30 material. Preferably, the assay strips are laminated dipsticks such as described in U.S. Patent No. 5,500,375.

In a preferred embodiment, this device includes holder having a stem portion and a funnel portion. In one embodiment, the holder can be a double sheet of plastic that is laminated together at the stem portion. For example, the holder can be made of polyethylene or another clear, flexible plastic. The funnel portion is sealed on the edges to form lateral seals that extend down the stem portion. Thus, an open top collection "funnel" is formed in the funnel portion, which is in fluid communication with the stem portion. At the neck of the device, or the juncture of the funnel portion and stem portion, is the processing and metering pad. The pad is preferably an absorbent pad or sponge which serves to filter oral debris and mucopolysaccharides from the sample. It can be formed of any suitable material, preferably of a fibrous nature, most preferably a material such as a cellulose or cellulose derivative. In some embodiments, the material may be charged or contain substances which effect separations or passage through the filter. For example, the pad may also contain buffers and reagents such as dissociating or mucolytic agents and surfactants which may be required. The pad further serves to meter the amount of sample which is transferred to the assay strip. This is accomplished by selection of the size, shape, porosity and composition of the pad, which can be adjusted as necessary to optimize separation and metering of sample volume.

The device includes one or more assay strips. These may be of the same or different materials, providing for measurement of multiple analytes in the sample. The assay strip extends from the pad into the stem portion. The assay strip includes a carrier which supports a membrane which is in liquid communication with the pad. The assay strip can be any chromatographic assay strip but is preferably designed as described in U.S. Patent Nos. 5,500,375 and 5,710,009. In the most preferred embodiment, the membrane is a nitrocellulose strip which includes a sample application region adjacent to the processing or metering pad. The membrane further includes a mobilization zone having immobilized thereupon mobilizable labelled reagent, for example. The membrane also includes one or more trap zones. Three contain various reagents for capturing unreacted analyte or labelled analyte, for example, depending upon

the design of the assay. In this preferred embodiment, the membrane further includes one or more detection zones which may be the same as one or more of the trap zones. The section of the holder overlying the detection zones is preferably clear so that the results of the assay can be read.

5    **IV. Other Immunoassays**

The apolipoproteins in the saliva can be measured using any of a number of different assays, including ELISA and automated immunoturbidimetric assays, as well as dipsticks made using conventional technology.

*ELISA*

10    In ELISA, sample is placed in separate wells in microtiter plates and allowed to adsorb to the wall of the wells. The wells are then treated with a blocking agent, such as bovine serum albumin or nonfat milk proteins, to cover areas in the wells not bound by antigen. Antibody is then added in an appropriate buffer to the well, in one or more concentrations and the microtiter  
15    plate incubated under conditions adequate to allow the antibody to bind the antigen adsorbed on the wall of each well. The presence of antibody bound to antigen (i.e., Apo A1, Apo B, etc.) in a well can then be detected using a standard enzyme-conjugated anti-antibody which will bind antibody that has bound to apolipoprotein in the well. Wells in which antibody is bound to  
20    antigen are then identified by adding a chromogenic substrate for the enzyme conjugated to the anti-antibody and color production detected by an optical device such as an ELISA plate reader.

Other detection systems can also be used, for example, a biotin-streptavidin system. In this system, one of the antibodies (either the antibody  
25    immunoreactive with the apolipoprotein or the antibody immunoreactive with the specific antibody) is biotinylated. The nonbiotinylated antibody are incubated with wells coated with the apolipoprotein or lipoprotein antigen. Quantity of biotinylated antibody bound to the coated antigen is determined using a streptavidin-peroxidase conjugate and a chromogenic substrate.

30    Antibodies can alternatively be labeled with any of a number of fluorescent compounds such as fluorescein isothiocyanate, europium, lucifer

yellow, rhodamine B isothiocyanate (Wood, P. In: Principles and Practice of Immunoassay, Stockton Press, New York, pages 365-392 (1991)) for use in immunoassays. In conjunction with the known techniques for separation of antibody-antigen complexes, these fluorophores can be used to quantify 5 apolipoprotein. The same applies to chemiluminescent immunoassay in which case antibody or apolipoprotein can be labeled with isoluminol or acridinium esters (Krodel, E. et al., In: Bioluminescence and Chemiluminescence: Current Status. John Wiley and Sons Inc. New York, pp 107-110 (1991); Weeks, I. et al., Clin. Chem. 29:1480-1483 (1983)). Radioimmunoassay (Kashyap, M.L. et 10 al., J. Clin. Invest. 60:171-180 (1977)) is another technique in which antibody can be used after labeling with a radioactive isotope such as  $^{125}\text{I}$ . Some of these immunoassays can be easily automated by the use of appropriate instruments such as the IMX<sup>TM</sup> (Abbott, Irving, TX) for a fluorescent immunoassay and Ciba 15 Corning ACS 180<sup>TM</sup> (Ciba Corning, Medfield, MA) for a chemiluminescent immunoassay.

#### *Immunoprecipitation*

Immunoprecipitation is another means of identifying small amounts of protein in a complex mixture by its interaction with antibody. The amount of antigen present can be determined by changes in turbidity of a solution using 20 optical detection means such as a spectrophotometer, or the precipitate isolated and measured by detection of label on the antibody, typically using ELISA, measurement of a fluorescent label or measurement of a radiolabel. In those cases where the antibody does not precipitate antigen, precipitation may be enhanced through the use of a second anti-antibody or a second antibody 25 immunoreactive with the same antigen.

For example, in an immunoturbidimetric assay for LDL, one preferably would use a single monoclonal antibody capable of precipitating exclusively LDL. Single Monoclonal antibodies generally do not precipitate the antigens they are immunoreactive with. Accordingly, two or more Monoclonal 30 antibodies immunoreactive with the same antigen can be used to precipitate the antigen. For quantitation of LDL, one would use two monoclonal antibodies

which are specific for LDL. Useful antibodies include HB3cB3 combined with another antibody such as WbA53aC1-A6, described by Koren, et al. Biochemistry 26, 2734-2740 (1987). This results in immunoprecipitation of LDL without affecting other plasma lipoproteins such as VLDL and HDL.

5        In addition, the above-described sandwich method can be used to detect any blood protein of interest in a particular sample, provided, as described above, that either two distinct Monoclonal antibodies are available which do not interfere with each other's binding to the particular protein, or one MAb and a polyclonal antibody are available for the particular protein and the MAb is 10      allowed to bind to the particular protein before the polyclonal antibody.

As noted above, anti-LDL Monoclonal antibodies, such as HB<sub>3</sub>cB<sub>3</sub>, are useful for quantification of LDL-cholesterol in antibody-antigen precipitation techniques and enzyme-linked immunosorbent assays (ELISA). For example, in a precipitation method the anti-LDL MAb is added to human serum or plasma 15      and allowed to bind to LDL. The immune complex of LDL bound to anti-LDL MAb is then precipitated by mixing in an excess amount of protein A or an anti-mouse IgG polyclonal antibody. Precipitation of the complexes is enhanced by centrifuging the mixture and then discarding the supernatant. The precipitate containing LDL is then washed and dissolved in 8 M urea in PBS or treated with 20      detergents such as Triton X-100 and cholic acid (Sigma, St. Louis, Mo). This is followed by determination of LDL-cholesterol using an enzymatic assay for cholesterol (Sigma, St. Louis, MO).

Antibodies can be bound to a solid phase material for use in assays described herein. Various types of adsorptive materials, such as nitrocellulose, 25      Immobilon<sup>TM</sup>, polyvinylidene difluoride (all from BioRad, Hercules, CA) can be used as a solid phase material to bind the anti-lipoprotein antibodies. Other solid phase materials, including resins and well-plates or other materials made of polystyrene, polypropylene or other synthetic polymeric materials can also be used. In the preferred embodiment for assaying lipoprotein concentrations, 30      pieces or strips of these materials are coated with one or more antibodies, or functional fragments thereof, directed against specific epitopes of HDL, LDL,

other lipoproteins, or apolipoproteins for use in patient samples. Such strips are referred to herein as "dipsticks". The dipsticks may also be attached to one end of a longer strip of a solid support material, such as plastic, which can serve as a handle for dipping a dipstick into a solution or sample. The plastic handle can 5 also serve as a tether so that multiple dipsticks can be attached to a common support. Such a multi-strip design may be particularly useful in a set-up for testing multiple lipoproteins and/or apolipoproteins simultaneously.

Although various sizes of dipsticks are possible, typically, pieces of the solid phase material that are coated with antibody have the general dimensions 10 of 0.5 cm x 0.5 cm and can be attached to the longer solid support strips having general dimensions of 0.5 cm x 5 cm. Such dimensions permit an accurate determination of lipoprotein or apolipoprotein levels in as little as 100  $\mu$ l of sample.

The present invention will be further understood by reference to the 15 following non-limiting examples.

**Example 1: Demonstration of the presence of Apolipoproteins in Saliva**

**A. Polyacrylamide gel electrophoresis**

Sample preparation

Fresh saliva samples were collected into test tubes and analyzed within 20 30 minutes or were kept overnight at 4C with 0.1% sodium azide. Serum samples were used as molecular weight markers; these were kept frozen and were thawed before analysis. Lipoproteins, HDL and LDL and apolipoproteins, ApoAI and ApoB from human plasma were from Calbiochem, La Jolla, CA. Before electrophoresis all samples were treated with 2.5% sodium dodecyl 25 sulfate (SDS) and incubated for 5 minutes in boiling water bath. In another variation of above treatment, the incubation mixture contained also 1% dithiothreitol (DTT). Bromophenol blue indicator was added to samples prior to electrophoresis.

Electrophoresis

30 Electrophoresis was performed with Phast System (Pharmacia Biotech, Piscataway, NJ) using manufacturers' protocols and materials. The

polyacrylamide gel at a pH 4-15 gradient and buffer system consisted of 0.2 M Tris buffer, pH 8.1 with 0.55% SDS. Pretreated samples were applied to gels and gels were subjected to automated separation according to the protocol provided by Pharmacia. After completion of electrophoresis, gels were stained 5 with Coomassie Blue R-350 and then destained and analyzed.

### Results

Results from electrophoresis of unreduced samples were as follows: The HDL sample and ApoAI sample migrated as single bands. Relative mobility (Rf), measured with reference to bromophenol blue marker was 0.94 for each 10 one. Rf for LDL sample was 0.24 and for ApoB sample was 0.25. The serum sample showed three bands at the region relative to HDL (Rf were 0.97; 0.94; and 0.88) and one band at the region relative to LDL (Rf=0.24). Saliva samples showed two bands at the region relative to HDL (Rf were 0.94 and 0.91) and one band at the region relative to LDL (Rf=0.24-in one case Rf=0.22).

15 Electrophoresis for DTT reduced samples showed HDL and ApoAI as single bands with Rf=0.85 for each. LDL appeared as two bands (Rf 0.26 and 0.22) and ApoB was a single band (Rf=0.26). Serum sample showed two bands at the region relative to HDL (Rf 0.82 and 0.79) and no sharp bank in LDL area. Saliva samples showed a single band at the region relative to HDL (Rf-0.82 for 20 each saliva sample) and no sharp band at the region relative to LDL.

Protein bands were only visible on gels when freshly collected saliva samples were analyzed.

### **B. Western Blotting.**

#### Protein Blotting

25 Protein blotting was done essentially according to the Phast System™ Development Technique File 220: Nitrocellulose (supplied with the Phast System unit) was prewetted with 1 X PBS. Upon completion of electrophoresis the nitrocellulose was placed on top of the gradient gel and the temperature was increased to 70°C and diffusion-mediated transfer proceeded for 20-30 minutes.

30 The nitrocellulose was incubated with shaking for 1 hour at RT followed by washing for 5 minutes in wash buffer (WB, 1 X PBS, 0.05% TWEEN®20).

The blot was then incubated with shaking for 30-60 minutes with a 1:1000 dilution of mouse anti-apolipoprotein B (monoclonal W<sub>a</sub>B<sub>2</sub>bD<sub>6</sub>, immunoreactive with ApoB independent of lipid content, obtained from Dr. Eugen Koren, Oklahoma Medical Research Foundation, Oklahoma City, OK) in 10mg/ml BSA, 1 X PBS. After a 5 minute wash in WB the gel was incubated for 30-60 minutes at room temperature (RT) with shaking with a 1:500,000 dilution of horseradish peroxidase (HRP) goat anti-mouse conjugate (Jackson ImmunoResearch; West Grove, PA) and subsequently washed 6 times. The blot was submerged in chemiluminescent development reagent SUPERSIGNAL BLAST<sup>TM</sup> (Pierce; Rockford, IL) for five minutes, placed in a transparent laminate, exposed to BIOMAX<sup>TM</sup> MR-2 film (Kodak; Rochester, NY for 10 seconds and developed with KODAK<sup>TM</sup> developer and fixer as per manufacturer's instructions.

### Results

Monoclonal anti-apolipoprotein B stained material of very high molecular weight was observed for both purified Apolipoprotein B and purified LDL. Each of these sample showed a faint band at the approximately 60kd site. In saliva the predominant staining was seen at approximately 60kd mw. When Apolipoprotein B was added to the saliva sample and incubated for 1 hour at room temperature prior to electrophoresis, a decrease in the amount of the high molecular weight band and a distinct increase in the immunoreactive material at approximately 60kd mw was observed, indicating that Apo B is degraded by the saliva enzymes.

Serum samples generated three bands that reacted with anti-Apo B, a double band at very high molecular weight which was also seen in purified LDL, and an approximately 60kd and possibly 45kd mw fraction. Purified LDL showed only a very high molecular weight band. Apo A1 was present in saliva in the 30kd serum form. Apo B immunoreactive with antibody was present in an approximately 60kd form: When LDL or ApoB was added to saliva and incubated the same approximately 60kd immunoreactive form was generated, indicating that the degradation is by saliva enzymes.

The levels of ApoA1 and ApoB measured in saliva by Western blot corresponded to about 1/50<sup>th</sup> of the amount seen in serum.

**Example 2: Correlation of ApoA:ApoB Saliva Levels with  
5 ApoA:ApoB Serum Levels.**

Sample Collection

*Saliva:* Stimulated and unstimulated saliva was collected and analyzed. Saliva flow was stimulated by asking the individual to suck either a lemon or a super mint. Stimulated saliva was collected by stimulating the same individual  
10 five minutes after collection of unstimulated saliva. Saliva samples were immediately filtered through a serum separator and then chilled in an ice water bath. Saliva samples were assayed within three hours of collection.

Stimulated saliva provided superior correlation. The super mint gave superior results and was more acceptable to saliva donors.

*15 Serum:* Serum was collected from 12 fasting subjects, immediately frozen and was thawed immediately before testing.

Analysis of Serum Lipids

Serum samples were analyzed for serum lipids on the Roche Cobas Mira Automated Chemistry Analyzer (software version 8735; Roche Diagnostic System, Nutley, NJ). Roche Apo A-1 and Apo B reagents and apolipoprotein standards were used to set up the calibration curve for determination of Apo A-1 and B in both serum and saliva samples. Roche cholesterol reagent and calibrator serum were used to determine the level of total cholesterol. Roche Unimate HDL direct HDL-Cholesterol calibrator and HDL direct calibrator  
20 were used to determine the level of HDL cholesterol. Roche triglycerides reagent and calibrator were used to determine the level of VLDL cholesterol, which is one-fifth of triglycerides. LDL cholesterol was determined as the difference of the determined sum of the VLDL and HDL cholesterol from total cholesterol.  
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30 For each of the assays, assay accuracy was monitored by comparison of experimental values to the published values for Liquichek and Lyphochek

control levels I and II (Bio-Rad; Hercules, CA) and for Cardiolipid and Apolipoprotein control levels I and II (Sigma Diagnostics; St. Louis, MO). Assay precision was estimated by determining average %CV of the same samples on successive days using Roche Reagents and additionally assayed for 5 ApoA1 and ApoB by Elisa Immunoassay of Lipoproteins. The correlation of the Roche serum assay with the Elisa was used to demonstrate correlation of the assays.

ELISA for Apo A-1 and B in Saliva:

Microtiter plates were coated overnight at RT and 100 microliters per 10 well of Apo A-1 or B from Intracell, Issaquah, WA added. Wells were blocked with 1% BSA/PBS for 2 hrs at RT. 10 microliters of saliva sample was incubated for 15 minutes at RT with 90 microliters of either rabbit polyclonal anti ApoA-1 (antibody provided by E. Koren, San Francisco) at 1:30,000 dilution or a 1:25,000 dilution of goat polyclonal anti ApoB (also provided by E. 15 Koren, OMRF). After a thirty minute incubation RT, plates were washed twice with 0.1% BSA/PBS. 100 microliters of HRP goat anti rabbit (ApoA-1 determination) or HRP rabbit anti-goat (ApoB determination) (Jackson ImmunoResearch was added with shaking at RT for 30 minutes). This was followed by two washes with BSA/PBS and followed by the addition of 150 20 microliters of 3, 3', 5, 5' tetramethyl-benzidine (TMB) for 15 minutes with shaking. The colorimetric reaction was stopped with 2 N sulfuric acid and plates were read on a Titertek Plate Reader at 450 nm to determine final absorbance. Absorbance readings were converted to mg/dL of ApoA-1 and B by reference to a calibration curve for ApoA-1 and B constructed from different 25 dilutions of a serum sample pool. ApoA-1 and B values for the undiluted sample pool were determined from the Roche autoanalyzer assay.

Elisa for albumin

Microtiter plates were coated overnight at RT with 100 microliters per 30 well of human serum albumin (Sigma A1151, St. Louis, MO) at 2 microgram/ml. Wells were blocked with 1% BSA/PBS for 2 hrs at RT. 10 microliters of saliva at 1:10 and 10 microliters at 1:100 was preincubated for 15

minute at RT with 90 microliters of goat anti-human albumin A1:100K and then transferred to the plate. After a thirty minutes incubation at RT, plates were washed twice with 0.1% BSA/PBS. 100 microliters of HRP rabbit anti-goat (Jackson ImmunoResearch) was added with shaking at RT for 30 minutes. This  
5 was followed by two washes with BSA/PBS and followed by the addition of 150ul of 3, 3' 5, 5' tetramethyl-benzidine (TMB) for 15 minutes with shaking. The colorimetric reaction was stopped with 2N sulfuric acid and plates were read on a Titertek Plate Reader at 450nm to determine final absorbance.  
10 Absorbance readings were converted to mg/dL of albumin by reference to a calibration curve constructed from different dilutions of a Sigma Human serum albumin.

### Results

Lipoproteins in serum and saliva samples competed with lipoproteins coated on plates for anti-apolipoprotein antibody binding, as shown by Figure 1.  
15 With increasing amount of lipoprotein in the sample, less antibody bound to the plate and decrease in signal was observed. Seven saliva samples exhibited different levels of measured apolipoproteins. The level of Apo A1 measured by ELISA in saliva samples was less than 10% of the expected serum level of Apo A1. The level of ApoB measured by ELISA in saliva samples was less than 1%  
20 of the serum level of Apo B.

The correlation between the values measured in serum samples using the commercially available Roche assay and the ELISA for Apo A and Apo B is shown in Table 1. The correlation between the values measured in serum samples for Apo A1 and Apo B by the Roche assay and serum LDL and HDL measured by the ELISA is shown in Table 2.  
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The correlation between Saliva Apo B/A1 and Serum Apo B/A1 measured by ELISA is shown in Table 3. In stimulated saliva samples, albumin is secreted in the same manner as the Apo B. As a result, albumin can be used to correct for dilution of the saliva sample. Apo B levels in saliva and Apo B levels normalized for dilution by reference to albumin correlate highly with serum Apo B and LDL levels.  
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The correlation coefficient for the Apo A:Apo B ratio obtained with stimulated saliva is not as high as the correlation coefficient in unstimulated saliva's when expressed in a Apo A/Apo B ratio did not correlate as well as unstimulated saliva. Apo A lipoprotein levels correlate  
5 highly  $r = 0.95$  and  $0.92$  with Apo B levels whether expressed as mg/dL or normalized to saliva albumin Figure 1a and b. In contrast the serum ApoA:B levels for these same samples had  $r =$  less than  $0.04$ . Thus it appears that during stimulated and (unstimulated) saliva flow Apo B secretion increases in relation to the amount of ApoA-1 and is on the order of three to four times as much Apo  
Q 10 ~~Figures 2a and 2b~~ <sup>Figures 2a and 2c</sup> B as Apo A, as shown in ~~Figures 2a and 2b~~, in contrast to the serum Apo B/Apo A ratio, which ranges from  $0.36$  to  $1.4$ . Thus, there is much higher Apo B:Apo A ratio than observed in serum. This could reflect the selectively higher secretion of Apo B in saliva or may be an artifact reflecting that the partially fragmented Apo B is more immunoreactive than the intact B protein.

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Table 1. Correlation (r) of the Roche Serum Assays with the Serum ELISA for Apo A and Apo B.

<u>Roche Values</u>	<u>ELISA</u>	<u>ELISA</u>	<u>ELISA</u>
	<u>Apo A1</u>	<u>Apo B</u>	<u>Apo B/A1</u>
ApoA1	0.81		
ApoB		0.94	
ApoB/A1			0.93

Table 2. Correlation (r) of Roche Serum Apo A1 and Apo B with Serum LDL and HDL Measured by ELISA.

	<u>LDL</u>	<u>HDL</u>	<u>LDL/HDL</u>
ApoA1		0.95	
ApoB1	0.98		
ApoB/A1			0.99

Table 3. Correlation (r) of Saliva ApoB/A1 with Serum ApoB/A1 Measured by ELISA.

<u>Saliva</u>	<u>Serum</u>	<u>Serum</u>	<u>Serum</u>
	<u>Apo B/A1</u>	<u>Apo B</u>	<u>LDL</u>
ApoB/A1	0.75		
ApoB/Albumin		.88	.82

Example 3: Detection of saliva levels of ApoB and ApoA-1 in competitive Format Immunochromatographic Strips.

Preparation of Immunochromatographic Strips

5 micron Nitrocellulose membrane (Millipore, Burlington, Mass.) was 15 coated with human HDL or LDL from Calbiochem, La Jolla, CA at a concentration of 2 mg/ml in an amount of 3 microliters/3 mm wide strip using a Camag Linomat IV (CAMAG, Switzerland). Gold particles were coated with

anti-ApoA1 with the monoclonal LpalHB4 (American Type Culture Collection, Rockville, MD), or with anti-ApoB with WaB2bD6 (Dr. Koren, OMRF).

Preparation of Samples

To assay, serum samples were diluted 1:2000, 1:600, 1:100, 1:75, 1:25  
5 and 1:10 50 into PBS. 50 microliter of sample was pipetted into a test tube containing 5 microliters of gold conjugate and 5 microliter of 5% BSA (Sigma, St. Louis, MI). The sample was vortexed and a strip was inserted in the tube. When the fluid had migrated to the end of the strip, the strip was removed from the tube, allowed to dry and the intensity of the band was measured using a  
10 Graytag D19C/D196 remission Densitometer (Greytag, Switzerland).

Results

Figure 3a and Figure 3b shows that color intensity was strongly inhibited by a 1:2000 dilution of serum and 1:100 dilution of Apo B. There was a good dose dependent inhibition of binding. Since saliva has an average of 1:50<sup>th</sup> of  
15 the amount of Apo A1 and at least that amount of ApoB, the results demonstrate that the assay of Apo A1 and Apo B in saliva proteins is amenable to quantitative immunochromatographic detection and could be adapted to the Serex reland format described in U.S. Patent Nos. 5,451,504, 5,500,375, and 5,710,00.

20 Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art and are intended to come within the scope of the appended claims.